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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This project takes Drosophila NF1 mutants and mouse *Nf1* mutants as models to investigate NF1-dependent regulation of the cAMP pathway. The study is intended to expand the knowledge of the genes that contribute to NF beyond the GAP-related domain in NF1. In the last year, our work has been mainly focused on two aspects. First, the effort has been devoted to establish biochemically how G-protein-dependent activation of adenylyl cyclase (AC) activity is regulated by NF1 in Drosophila. Second, whether a similar biochemical regulation of AC activity can be identified in vertebrates. These studies have led to a finding that G-protein-dependent activation of AC consists of two components: one is classically described NF1-independent one while the other is NF1-dependent. Accumulated evidence from our last year's effort support that a similar NF-dependent mechanism can be observed in vertebrates. We are continuing to pursue a molecular understanding of this regulation and whether this pathway contributes to pathogenesis of NF1.

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Introduction:

The proposed research aims to investigate how the neurofibromatosis 1 (NF1) protein regulates adenylyl cyclase activity in Drosophila and in mice. This pathway has been shown to be critical for mediating a neuropeptide response, cell-size control, and learning and memory in *Drosophila*. Two specific aims have been proposed, including (I) biochemical analysis of how G-protein dependent activation of adenylyl cyclase (AC) is affected by NF1 and whether small G protein Ras is involved in regulation and (II) biochemical analysis of NF1-regulated AC activity in mice. In the previous report, we have shown that NF1-regulated AC activity can be observed in the mouse (Tong et al., 2002, Nature Neuroscience). For last yeas, we have mainly examined another newly identified mechanism for activation of AC, i.e. Ras stimulation of AC. This study has been conducted in Drosophila. The results are summarized below.

Body:

1. NF1-dependent Ras activation of AC activity.

In the classic point view, AC can be stimulated by heterotrimeric G protein activation and by Ca2+/CaM. Our earlier work has demonstrated that activation of AC via heterotrimeric G protein includes two component: direct activation by Gα (classic) and NF1-dependent G protein activation. Here we have evidence to show that Ras is also capable of stimulating AC activity in an NF1-dependent manner. Following results were obtained mainly in the last year.

(a) Human H-Ras and K-Ras were able to stimulate AC activity in Drosophila. Application of purified H-Ras or K-Ras to the membrane fraction extracted from head tissues increased AC activity significantly and the increase was abolished in two NF1 mutant alleles, $NF1^{P1}$ and $NF1^{P2}$.
(b) Purified GAP-related domain (GRD) of human NF1 was also able to stimulate AC activity. We interpreted this observation as to that applied GRD bound with free Ras in the extracts and then stimulated AC activity. This was supported by the observation that GRD with mutations that reduced either GAP activity or Ras binding attenuated the stimulation. Moreover, the normal GRD failed to stimulate AC activity in Ras mutants.
(c) We have previously shown that a neuropeptide, pituitary adenylyl cyclase-activating polypeptide (PACAP), is able to stimulate NF1-dependent G protein activation of AC. To examine cellular function of this Ras stimulation of AC activity, we have examined a number of growth factors. It is known that growth factors activate Ras. We found that epidermal growth factor (EGF) is capable of stimulating AC activity. We are now investigating whether this EGF-stimulated CA activity is indeed mediated via EGF receptors in Drosophila and via NF1-dependent Ras activation of AC.

2. Site-directed mutagenesis.

We are continuing investigation of effects of clinical-relevant mutations. We have shown in the year before that expression of human NF1 (hNF1) in Drosophila is able to rescue the mutant phenotype of small body size. We now have shown that expression of hNF1 is also able to rescue other fly mutant phenotypes, including the learning defect and abnormal circadian rhythm. We have now generated

transgenic flies that carry the clinical-relevant mutant hNF1 gene. Examination of four mutations, with two located within GRD and two outside GRD, all appeared to rescue the learning and body size phenotypes. This suggests that clinical-relevant mutations do not affect G protein activation of AC and Ras is not required for G protein activation of AC. We are examining how Ras-related phenotypes, such as circadian, may be affected by these mutations.

Key Research Accomplishments:

(1) Ras is able to regulate AC activity in NF1-dependent manner.
(2) EGF stimulates AC activity.
(3) Ras is not required for G protein activation of AC
(4) NF1-dependent G protein activation of AC may not contribute to pathogenesis of NF1.

Reportable Outcomes:

1. Presentations in NF meeting held at Aspen in 2002.

Conclusion:

Over last year, we have demonstrated that Ras is capable of stimulating AC activity in an NF1 dependent manner. This pathway may be involved in mediating growth factor signaling. We have shown that NF1-dependent G protein activation of AC may not contribute to pathogenesis of NF1. We will continue our efforts to determine molecular mechanisms by which NF1 regulates AC activity and whether and how such signal transduction pathway contributes to pathogenesis of NF1.

Appendices:

1. Tong, J., Hannan, F., Zhu, Y., Bernards, A and **Zhong, Y.** (2002) Neurofibromin regulates G Protein-Stimulated adenylyl cyclase activity. *Nature Neuroscience,* 95-96

Neurofibromin regulates G protein–stimulated adenylyl cyclase activity

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Neurofibromatosis type 1 (NF1) is a dominant genetic disorder characterized by multiple benign and malignant nervous system tumors, and by learning defects in 45% of children with *NF1* mutations. Studies of neurofibromin, the protein encoded by *NF1*, have focused on its functions in tumorigenesis and regulation of Ras activity; however, *Drosophila NF1* regulates both Ras and cyclic AMP (cAMP) pathways. Expression of a human *NF1* transgene rescued cAMP-related phenotypes in NF1 mutant flies (small body size and G protein–stimulated adenylyl cyclase (AC) activity defects), and neuropeptide– and G protein–stimulated AC activity were lower in $Nf1^{-/-}$ as compared to $Nf1^{+/-}$ mouse brains, demonstrating that neurofibromin regulates AC activity in both mammals and flies.

Genetic analysis confirms the role of neurofibromin in tumorigenesis in mouse[1,2] and in learning and memory in mouse[3,4] and *Drosophila*[5]. Mounting evidence suggests that neurofibromin may be involved in functions besides Ras regulation. First, several hot spots for point mutations identified in individuals with NF1 occur outside the GAP (GTPase activating protein)–related domain[6]. Second, Ras inhibitors can rescue only some phenotypes in *NF1*-deficient cell lines[7]. Third, neurofibromin binds another protein, syndecan[8], in addition to Ras. Fourth, *Drosophila* NF1 regulates G protein–dependent AC activity, which is important for learning and memory[5], a neuropeptide response[9] and regulation of body size[10]. Also, *Drosophila* NF1 regulates Ras activity *in vivo*, as reduced Ras activity rescues a circadian rhythm defect in *Drosophila NF1* mutants[11].

We first examined whether the human *NF1* gene (*hNF1*) could function in flies, focusing on the small body size and AC activity phenotypes. The fly NF1 protein is 60% identical to human neurofibromin[10]. Two *NF1* mutations cause smaller body size: $NF1^{P1}$, a deletion of the *NF1* locus and several adjacent genes, and $NF1^{P2}$, a P-element insertion[10]. This phenotype is rescued by increasing cAMP but not by attenuating Ras activity[10]. Expression of the *hNF1* transgene in all cells in *NF1* mutant flies, under control of yeast Gal4–upstream activating sequences (*UAS-hNF1*), rescued the small-body-size phenotype, as measured by pupal length using two different Gal4 driver lines (**Fig. 1a**)(**Supplementary Methods**, available on the *Nature Neuroscience* web site). Rescue was almost complete in $NF1^{P2}$ but only partial in $NF1^{P1}$. Incomplete rescue of a neuropeptide response is also seen in $NF1^{P1}$ (ref. 9). G protein–stimulated AC activity is lower than normal in *Drosophila NF1* mutants and can be rescued by acute expression of a *Drosophila NF1* transgene[5]. We found that expression of the *hNF1* transgene controlled by the *Gal4–UAS* system also rescued the AC-activity defect in *NF1* mutant flies (**Fig. 1b**). Thus, human neurofibromin can directly regulate cAMP signaling in *Drosophila*.

Next, we looked at G protein–stimulated AC activity in homozygous knockout mice ($Nf1^{-/-}$). Because $Nf1^{-/-}$ mice die at embryonic day 13.5 (E13.5)[12], assays were restricted to E12.5 frontal brain extracts. The magnitude of AC activity in control extracts was similar among wild-type, $Nf1^{+/-}$ and $Nf1^{-/-}$ mice (**Fig. 2a**). Among extracts stimulated with GTPγS, however, AC activity was significantly less in the $Nf1^{-/-}$ homozygous mutant than in $Nf1^{+/-}$ and wild-type mice (**Fig. 2a**), even though AC activity is limited in embryonic tissues (**Supplementary Fig. 1**, available on the *Nature Neuroscience* web site). In addition, cAMP concentration was significantly lower in $Nf1^{-/-}$ as compared to $Nf1^{+/-}$ embryos (**Fig. 2b**), supporting the observation of lower AC activity in the $Nf1^{-/-}$ embryos. We also examined the effect of the neuropeptide pituitary adenylyl cyclase–activating polypeptide (PACAP), as PACAP-induced modulation of K^+ currents is abolished in *Drosophila NF1* mutants[9]. PACAP-stimulated AC activity was similar in the three genotypes in $Nf1^{+/-}$ and wild-type mice but lower in $Nf1^{-/-}$ mice (**Fig. 2c**).

Considering the developmental abnormalities in $Nf1^{-/-}$ mice[12], the defect in stimulation may have resulted from reduced AC functionality. An adequate amount of AC was available in $Nf1^{-/-}$ mice, however, as forskolin stimulated AC activity equally in all genotypes (**Fig. 2d**). There may also have been reduced expression of G proteins in $Nf1^{-/-}$ mice; however, there was no difference in the

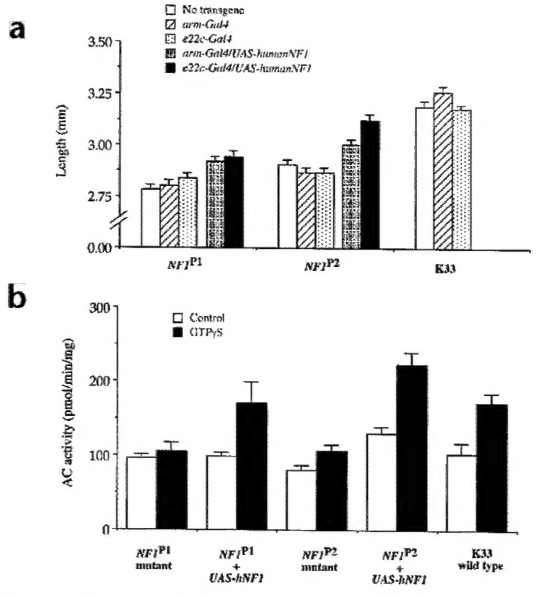

Fig. 1. Rescue of body-size phenotype and GTPγS-stimulated AC activity by human *NF1* transgene under Gal4–UAS control. (**a**) Pupal length is normal in control wild-type K33 flies and reduced in $NF1^{P1}$ and $NF1^{P2}$ flies. Global activation of the *UAS–hNF1* transgene using *e22c–Gal4* or *arm–Gal4* (in both *NF1* mutant backgrounds) significantly ($p < 0.001$) increased pupal length over values for *NF1* mutants (bars, mean ± s.e.m.; $n = 50$ for each genotype). (**b**) GTPγS-stimulated AC activity was assayed in fly head membranes. Significant stimulation was seen in wild-type K33 flies but not in *NF1* mutants. Activation of the *UAS–hNF1* transgene by either Gal4 driver line resulted in significant ($p < 0.01$) increases in GTPγS-stimulated AC activity ($n = 3, 3, 4, 4, 7$).

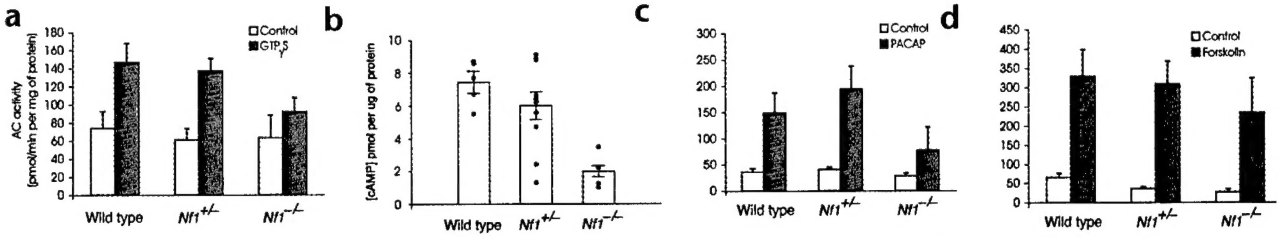

Fig. 2. Reduction in stimulated AC activity and cAMP levels in mouse *Nf1* knockout. **(a)** GTPγS-stimulated AC activity, assayed in E12.5 frontal brain membrane extracts, was lower ($p < 0.05$) in homozygous $Nf1^{-/-}$ mice than in both $Nf1^{+/-}$ and wild-type mice ($n = 9, 23, 13$) and was not significantly higher than in controls ($p > 0.3$). **(b)** Reduced cAMP concentration in $Nf1^{-/-}$ frontal brain compared to wild type ($p < 0.001$) and $Nf1^{+/-}$ ($p < 0.01$), and high variance in $Nf1^{+/-}$ embryos (6.97) compared to wild type (1.81) and $Nf1^{-/-}$ (1.27), as shown by data points from individual embryos (gray circles). Superimposed are mean ± s.e.m of cAMP concentration. **(c)** PACAP-stimulated AC activity is also reduced in $Nf1^{-/-}$ compared to wild type and $Nf1^{+/-}$ ($p < 0.01$, $n = 6, 20, 8$). **(d)** Forskolin-stimulated AC activity is normal in *Nf1* knockout compared to wild type and $Nf1^{+/-}$ ($n = 4, 22, 7$).

amount of the stimulatory Gα subunit present (**Supplementary Fig. 2**). To rule out any possible effect of dying embryos, AC activity was also assayed in one-month-old primary neuronal cultures. There was no difference in the growth or morphology of cultured neurons (**Fig. 3a**). As observed in *in vivo* assays, AC activity in control and forskolin-stimulated extracts were similar in all genotypes, whereas AC activity in GTPγS-stimulated extracts was significantly lower in the $Nf1^{-/-}$ genotype (**Fig. 3b**).

These results, revealing *NF1*-dependent regulation of AC activity in vertebrates, together with a study showing that *Drosophila* NF1 regulates Ras activity *in vivo*[11], indicate that *NF1* is conserved not only structurally but also functionally in *Drosophila*, mouse and human. The rescue of the fly *NF1* defects by expression of the human *NF1* transgene further supports this notion. In flies, this *NF1*-regulated AC activity is mediated chiefly *via* the *rutabaga*-encoded AC (Rut-AC)[5], which is the only AC known to be responsive to Ca^{2+}/calmodulin (CaM) in *Drosophila*[13]. In contrast, two types of AC, AC1 and AC8, are sensitive to Ca^{2+}/CaM in vertebrates[14]. It remains to be determined whether AC1 (which is homologous to Rut-AC), or AC8 or both are involved in mediating *NF1*-regulated AC activity. We saw no significant difference in mean AC activity or cAMP concentrations in heterozygous $Nf1^{+/-}$ mice as compared to wild-type embryos (**Figs. 2** and **3**). Thus, the NF1-regulated AC pathway may have more influence on phenotypes that require loss of heterozygosity than on clinical manifestations observed in heterozygous individuals, such as learning deficits. Activity of AC in postembryonic heterozygous $Nf1^{+/-}$ mice may show significant differences, however, given the larger G protein–stimulated AC activity observed at later stages of development (**Supplementary Fig. 1**). In addition, the variance in cAMP concentrations was much larger in heterozygous embryos (**Fig. 2b**), which may explain why learning deficits are not seen in all patients and mice[3,4].

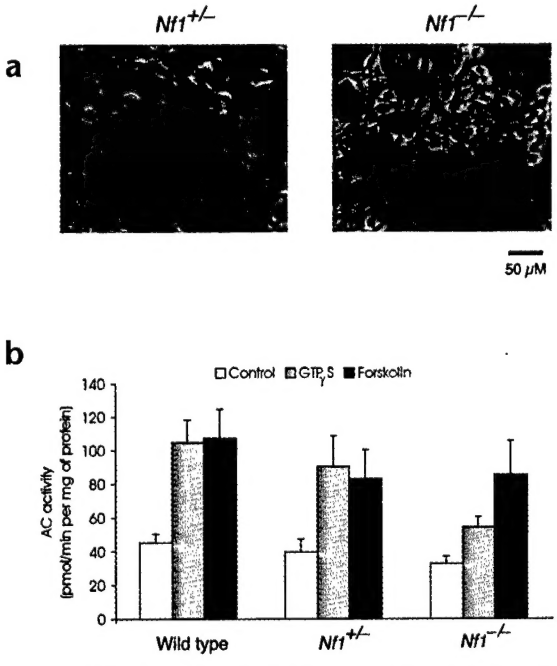

Fig. 3. Altered G protein–stimulated AC activity in primary embryonic neuronal cultures from mouse *Nf1* knockout. **(a)** No difference in growth or morphology was observed in $Nf1^{+/-}$ and $Nf1^{-/-}$ neurons. **(b)** GTPγS-stimulated AC activity is significantly lower in $Nf1^{-/-}$ mice compared to wild type ($p < 0.01$) and $Nf1^{+/-}$ ($p < 0.05$) ($n = 11, 11, 7$). GTPγS-stimulated AC activity is also significantly higher ($p < 0.05$) than in control unstimulated $Nf1^{-/-}$ mice. AC activity was similar among all genotypes in control and forskolin-stimulated extracts.

Note: Supplementary figures and detailed methods are available on the Nature Neuroscience web site (http://neurosci.nature.com/web_specials).

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Competing interests statement

The authors declare that they have no competing financial interests.

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